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2,4-DIAMINO-5-(3,5-DIMETHOXY-4-SUBSTITUTED)-BENZYL-PYRIMIDINES AS LIGANDS IN AFFINITY CHROMATOGRAPHY OF BACTERIAL DIHYDROFOLATE REDUCTASES *

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Summary

Inhibitors of the trimethoprim-type, bearing terminal amino, hydroxyl or carboxyl groups in position 4 of the benzene ring as well as methotrexate were coupled to either CH-Sepharose 4B, epoxy-activated Sepharose 6B or AH-Sepharose 4B, respectively. In contrast to the methotrexate-affinity gel, trimethoprim-like ligands retained bacterial but not mammalian dihydrofolate reductases. The affinity gels prepared with these ligands could be used for effective purification of bacterial dihydrofolate reductases (EC 1.5.1.3), but differed in their affinity for this enzyme.

Introduction

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) is an intensively studied enzyme because of its importance as a target site for chemotherapeutic agents such as methotrexate, trimethoprim or pyrimethamine, its comparatively low molecular weight and its tremendous species differences with respect to inhibition by various types of inhibitors [1].

This calls for rapid and effective purification methods. Most investigators therefore include an affinity step on the way to homogeneous preparations. The affinity column most commonly used is methotrexate-Sepharose, which binds all types of dihydrofolate reductase [2,3], however with the disadvantage that the binding of the enzyme is often so tight that rigorous elution methods have to be applied. Amethopterin or aminopterin coupled to soluble starch has

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also been used for affinity chromatography [3,4], as well as pteroyllysine [5], folate [6] and recently dihydrofolate [7], all attached to Sepharose. Folate has also been linked to bovine serum albumin, followed by covalent coupling to activated Sepharose [8]. Like other dehydrogenases, especially folate enzymes, dihydrofolate reductase can also be purified on Procion Red HE 3B-Sepharose [9].

The use of trimethoprim-type inhibitors of dihydrofolate reductase, which are reversible, competitive and specific, as affinity ligands, is described in this communication.

Materials and Methods

Chemicals. Epoxy-activated Sepharose 6B, AH-Sepharose 4B and CH-Sepharose 4B were purchased from Pharmacia (Uppsala). 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate was obtained from Serva (Heidelberg) and methotrexate from Lederle (Pearl River, NY, U.S.A.). All other chemicals used were reagent grade. The pyrimidine compounds used were prepared by Dr. I. Kompis, Dr. Rey-Bellet, Dr. Wick and Dr. Zanetti and the synthesis of some of them is reported elsewhere [10].

Coupling procedure. The coupling procedures were done as recommended by the manufacturer. The inhibitors containing terminal amino groups at position 4 were coupled to the carboxyl group of CH-Sepharose 4B using the carbodiimide or to epoxy-activated Sepharose 6B via a $-NH-CH_2-CHOH$ group. Compound No. IV, containing a hydroxyl group was similarly coupled to epoxy-activated Sepharose 6B via a $-O-CH_2-CHOH$ group. Compounds containing carboxyl groups were joined to the amino groups of AH-Sepharose 4B by carbodiimide coupling.

Enzymes and assay. Partially purified preparations of dihydrofolate reductase were obtained from *Escherichia coli* B or *E. coli* 114 (R 388) by sonic treatment of cells, a streptomycin sulfate precipitation step and treatment with $(NH_4)_2SO_4$ as described [11]. The dialyzed 50—90% saturated $(NH_4)_2SO_4$ fraction of *E. coli* B was used as the enzyme source in most cases. The rat liver enzyme was obtained similarly, but an initial acid-treatment step (to pH 4.5 with 1 N HCl and readjustment to pH 7.0 with 1 N NaOH) was carried out. The assay was performed as described [11]. 1 unit of enzyme is defined as the amount which catalyzes the production of 1 nmol tetrahydrofolate/min under the assay conditions.

Results and Discussion

Properties of the compounds used

We have observed that a wide range of structural changes in 2,4-diamino-5-(3,5-dimethoxybenzyl) pyrimidines are tolerated without losing the binding properties to bacterial dihydrofolate reductase. A large series of compounds of this type has been synthesized [10], many of which inhibited the enzyme at concentrations equal to or slightly higher than those needed for trimethoprim. Since trimethoprim itself cannot be attached direct to activated Sepharose in the required position, some of these derivatives were judged suitable for one-

step coupling procedures. Those reactions have become very convenient, since activated Sepharose preparations, containing different spacer groups, are commercially available. The compounds used for this study are listed in Table I. The type of activated Sepharose to which they were coupled is also indicated, as well as their inhibitory potency for the *E. coli* and rat liver dihydrofolate reductase in comparison to trimethoprim and methotrexate (given as those concentrations which inhibit the reaction by 50% [11]. They are all less potent inhibitors than trimethoprim and in contrast to methotrexate, binding to bacterial dihydrofolate reductase is reversible.

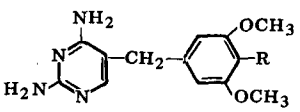
Coupling procedures

The total volume of the coupling mixture, containing the swollen gel, carbodiimide and ligand was 20—30 ml. In general, approx. 300 μmol of ligand were dissolved and coupled to 5 g of washed and swollen AH- or CH-Sepharose 4B with the aid of 200 mg carbodiimide at pH 4.5—6.0 by overnight incubation under gentle shaking at room temperature. Similarly, 290 μmol of substance No. I were directly coupled to 5 g of swollen epoxy-activated Sepharose 6B. The reaction was carried out at room temperature at pH 9—10 for 16 h. Because of limited substance only 38 μmol of compound No. III (which is only 10—15% of the amount theoretically to be coupled) and 103 μmol of compound No. IV were used for coupling to epoxy-activated Sepharose.

The resulting gels were washed thoroughly with 1 M ethanolamine, 0.1 M borate buffer (pH 8.0), 0.1 M acetate buffer (pH 4.0), 10 mM phosphate buffer

TABLE I

TRIMETHOPRIM-ANALOGUES USED FOR COUPLING TO DIFFERENT ACTIVATED SEPHAROSE PREPARATIONS AND THEIR INHIBITORY PROPERTIES



No.	R	Inhibition of dihydrofolate reductase (I_{50}, μM) from		Coupled to
		E. coli	rat liver	
I	$-NH_2$	0.024	700	epoxy-activated Sepharose 6B
II	$-NH_2$	0.024	700	CH-Sepharose 4B
III	$-CH_2NH_2$	0.6	>1000	epoxy-activated Sepharose 6B
IV	$-CH_2OH$	0.020	600	epoxy-activated Sepharose 6B
V	$-NHCOCH_2CH_2COOH$	0.029	2600	AH-Sepharose 4B
VI	$-NHCH_2COOH$	0.032	>1000	AH-Sepharose 4B
trimethoprim	OCH_3	0.009	500	—
methotrexate	—	0.002	0.006	AH-Sepharose 4B

(pH 7.0) on a funnel and finally poured into 15 × 100 mm or 15 × 200 mm columns.

Properties of the affinity gels

The amount of ligand which was bound covalently to Sepharose cannot easily be checked. With methotrexate, however, a bright yellow gel was obtained. Proof that the coupling step was successful was obtained by the fact that all gels retained the *E. coli* dihydrofolate reductase, whereas the corresponding enzyme from rat liver was not. The enzyme was applied as a dialyzed 50—90% $(NH_4)_2SO_4$ fraction in 10 mM phosphate buffer (pH 7.0). In most cases a small amount of dihydrofolate reductase appeared in the first fractions, but when more purified preparations were applied (e.g. after a gel filtration step) this was not the case.

The fact that bacterial dihydrofolate reductase was retained shows that those analogues coupled to the spacer by their amino-groups were at least partly bound by the amino group of the benzyl moiety; substitution of the amino groups of the pyrimidine ring would almost completely abolish the affinity for dihydrofolate reductase (this has been constantly found in our own and other laboratories) and hence ligands bound via the pyrimidine-amino groups would most probably not bind dihydrofolate reductase.

The affinity of the different ligands for the *E. coli* dihydrofolate reductase obviously varied. Gels obtained by coupling compounds Nos. I, IV and VI bound the enzyme weakly, since activity was gradually removed by increasing the ionic strength with 0.1, 0.5 or 1 M NaCl. Gels obtained by coupling compounds Nos. II, III and V bound the enzyme more tightly. In these cases dihydrofolate reductase could only be removed with folate or dihydrofolate in high salt. 2 mg/ml of folate in 10 mM phosphate buffer (pH 7.0) containing 0.5 or 1 M NaCl or sometimes 2 mg/ml of dihydrofolate in 0.5 M KH_2PO_4 were used for elution of the enzyme. It seems obvious from the different behaviour of these gels that the affinity for the enzyme is not strictly dependent on the potency of the inhibitor used as ligand but that the nature and length of the spacer group, as well as the amount of ligand bound is of importance. It may even seem that the more hydrophobic CNBr-activated spacer groups are more favourable for binding this enzyme than epoxy-activated groups. In order to remove dihydrofolate reductase from the methotrexate column completely, the pH also had to be increased to 8.0 with 10 mM Tris-HCl. In general 70—90% of the activity applied was recovered after the affinity step. With the gel obtained by coupling compound No. III it was also seen that activity could be removed specifically by trimethoprim (100 μg/ml or less) and also by triamterene, a less potent inhibitor of dihydrofolate reductase (I_{50} = 4 μM). Extensive dialysis is, however, required before testing the eluant in order to remove trimethoprim or any other inhibitor used for elution.

Repeated purification processes with slightly modified procedures yielded purification factors of several 100-fold starting with relatively crude preparations. The columns were used repeatedly for several months without apparent loss of efficiency.

The assumed configuration of the gels obtained by coupling compounds No. II and III, which were very effective and exhibit different spacer groups, are

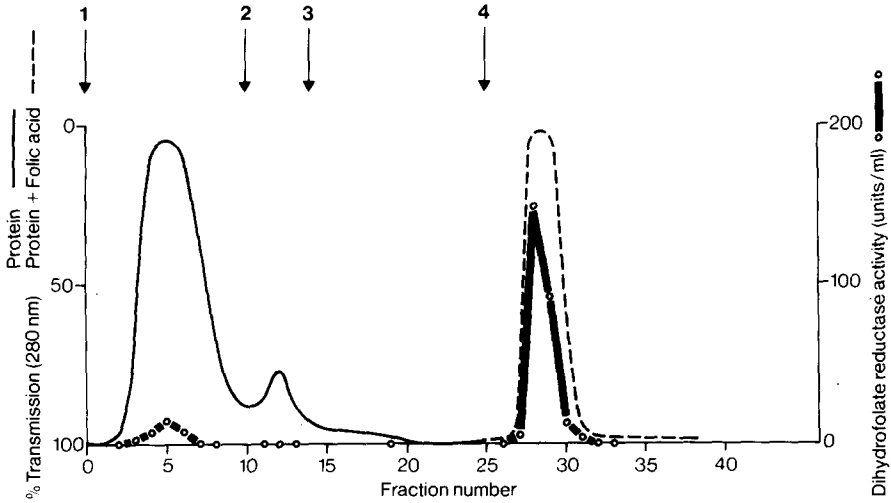
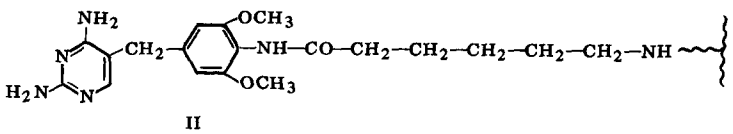
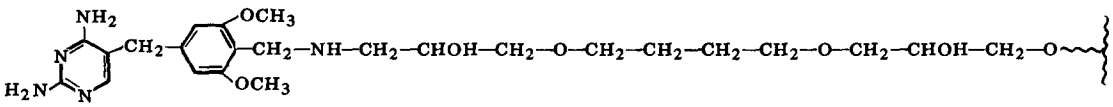


Fig. 1. Purification of the dihydrofolate reductase from *E. coli* B on affinity gel No. II. 1. Additions of 4 ml dialyzed 50—90% $(NH_4)_2SO_4$ fraction (approx. 2000 units) and subsequent wash with 10 mM phosphate buffer (pH 7.0). 2. 0.5 M NaCl wash. 3. 1 M NaCl wash. 4. Elution of enzyme by adding 3 ml of a 2 mg/ml folate solution in phosphate buffer, containing 0.5 M NaCl, and subsequent wash without folate, 5-ml fractions.

shown below. This indicates the assumed structure of Sepharose-bound



II



III

trimethoprim-like inhibitors, prepared by coupling to CH-Sepharose 4B (II) or epoxy-activated Sepharose 6B (III).

A representative purification procedure for the enzyme from *E. coli* B is given in Fig. 1.

An extract of *E. coli* 114 (R 388) which harbours an additional plasmid-determined and trimethoprim-resistant dihydrofolate reductase [12] was also applied to a column containing the bound compound No. III. Whereas the trimethoprim-sensitive (chromosomal) enzyme was retained, the resistant (plasmid) reductase was not. Both enzymes could easily be identified by the differences in molecular weight using gel chromatography and by the insensitivity of the plasmid enzyme for trimethoprim and other antifolates [12].

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